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Quantitative analysis of glycerol in dicarboxylic acid-rich cutins provides insights into Arabidopsis cutin structure

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ABSTRACT

Cutin is an extracellular lipid polymer that contributes to protective cuticle barrier functions against biotic and abiotic stresses in land plants. Glycerol has been reported as a component of cutin, contributing up to 14% by weight of total released monomers. Previous studies using partial hydrolysis of cuticle-enriched preparations established the presence of oligomers with glycerol-aliphatic ester links. Furthermore, glycerol-3-phosphate 2-*O*-acyltransferases (*sn*-2-GPATs) are essential for cutin biosynthesis. However, precise roles of glycerol in cutin assembly and structure remain uncertain. Here, a stable isotope-dilution assay was developed for the quantitative analysis of glycerol by GC/MS of triacetin with simultaneous determination of aliphatic monomers. To provide clues about the role of glycerol in dicarboxylic acid (DCA)-rich cutins, this methodology was applied to compare wild-type (WT) Arabidopsis cutin with a series of mutants that are defective in cutin synthesis. The molar ratio of glycerol to total DCAs in WT cutins was 2:1. Even when allowing for a small additional contribution from hydroxy fatty acids, this is a substantially higher glycerol to aliphatic monomer ratio than previously reported for any cutin. Glycerol content was strongly reduced in both stem and leaf cutin from all Arabidopsis mutants analyzed (*gpat4*/*gpat8*, *att1-2* and *lacs2-3*). In addition, the molar reduction of glycerol was proportional to the molar reduction of total DCAs. These results suggest "glycerol-DCA-glycerol" may be the dominant motif in DCA-rich cutins. The ramifications and caveats for this hypothesis are presented.

1. Introduction

Cutin is one of the two major extracellular lipid polyesters in higher plants. As the structural framework of plant cuticles, it is deposited at the outer surface of the primary cell wall. Together with the embedded and subtended waxes, this hydrophobic polymer covers the aerial surface of all land plants, and particularly leaf and stem epidermis, and therefore is one of the most abundant lipid polymers in nature. Cutin contributes to protective cuticle barrier functions against pathogen invasion and the control of gas, water and solutes fluxes, and plays a role in preventing organ fusion. Suberin, the other major lipid polyester, is deposited at the inner surface of the primary cell wall. It is most well known as a major constituent of tree bark, but is found in a variety of internal and external tissues and is also formed as a response to wounding, stress and abscission. Both cutin and suberin are fatty acid- and glycerol-derived polymers that are insoluble in water and organic solvents. Glycerol (**6**) (Fig. 1) was detected as a component of cork suberin in the late nineteenth century (Kügler, 1884), but it was identified as a component of cutin only recently (Graça et al., 2002). The fatty acids released through depolymerization can include simple fatty acids, ω-hydroxy fatty acids (ω-OHFAs) and α,ω-dicarboxylic acids (DCAs). Although C16 and C18 hydroxy fatty acids are usually the dominant cutin monomers (Kolattukudy, 1981), DCAs, originally considered as distinctive suberin monomers (Matzke and Riederer, 1991), have also been identified as major components in Arabidopsis stem and leaf cutins (Bonaventure et al., 2004; Franke et al., 2005), as well as *Brassica napus* leaf cutin (Bonaventure et al., 2004; Chen et al., 2011). The DCA-rich cutin is essential for the formation of stomatal ledges (Li et al., 2007a). Representative cutin monomers found in Arabidopsis are shown in Fig. 1 as **1**–**6**. Despite its well-documented monomer composition, the exact three-dimensional structure of cutin remains elusive.

The insoluble nature of cutin and suberin, and their integration

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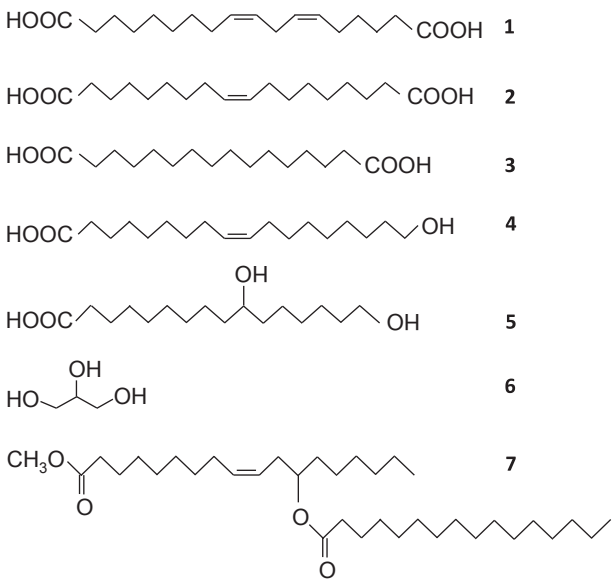

Fig. 1. Structures of the chemical entities described in text, including common Arabidopsis cutin monomers (**1**–**6**) and a synthesized lipid compound 12-palmitoyl-ricinoleate (**7**). Monomers **1**–**6** are analyzed by GC as methyl ester (for COOH groups) and acetyl ester (for OH groups) derivatives. Thus, for example, **4** is analyzed as methyl 18-acetyloxyoctadec-9-*cis*-enoate (methyl 18-acetyloxyoleate), and **6** as glycerol triacetate (triacetin). Collectively, monomers such as **1**–**3** are described as α,ω-dicarboxylic acids (DCAs), and monomers such as **4** and **5** as ω-hydroxy fatty acids (ω-OHFAs). Dihydroxypalmitates occur as a series of positional isomers, the major 10,16-isomer (**5**) being shown.

into the plant cell wall, makes structure determination of these polymers challenging. Through mild partial hydrolysis, monoacylglycerols have been identified from the isolated plant cuticles (Graça et al., 2002) and suberin enriched tissues (Graça and Pereira, 1997, 1999, 2000a; Graça and Santos, 2006). Glycerol-containing trimers, including those with DCA:ω-OHFA:glycerol stoichiometries of 1:0:2, 2:0:1, and 1:1:1, have also been identified from suberin (Graça and Pereira, 2000b; Graça and Santos, 2006). Such oligomer analyses define monomer bonding connectivity but do not produce a complete structural accounting. NMR spectroscopy is an alternative approach to providing a broad structural overview of cutin and suberin preparations, but details can be hard to finesse (Deshmukh et al., 2003; Fang et al., 2001; Stark et al., 2000; Yan and Stark, 2000). In this regard, genetic studies, and mutant analyses in particular, can be advantageous in allowing some inferences about overall polymer structure. For instance, CYP86B1 is a cytochrome P450 essential for the formation of C22:0 and C24:0 DCAs in suberin. Analysis of seed suberin composition in *cyp86b1* mutant established a significant molar portion (≥60%) of C22:0 and C24:0 fatty acids, challenging the traditional view of an extensive aliphatic domain in suberin (Molina et al., 2009). In the same study, the putative role of ester-linked ferulate in suberin lamellae structure was questioned using the *asft* mutant. Lack of this acyltransferase resulted in almost complete loss of suberin-associated ferulate but did not alter suberin lamellae ultrastructure.

In the past decade, significant progress has been made in cutin research using the model plant Arabidopsis. Enzyme families required for cutin biosynthesis have been identified. These include acyl-activating enzymes of LACS family, acyl-oxidizing enzymes of CYP86A subfamily, and acyltransferases of *sn*-2-GPAT family. Each of the three enzyme classes plays an essential role in the provision of cutin acylglycerol monomers, although some details of the process remain obscure (Pollard et al., 2008; Beisson et al., 2012). In particular, *LACS2* (Bessire et al., 2007; Schnurr et al., 2004), *CYP86A2* (or *ATT1*) (Molina et al., 2008; Xiao et al., 2004), and *sn*-2-*GPATs* 4 and 8 (Li et al., 2007a; Yang et al., 2010; Yang et al., 2012) are clearly important for Arabidopsis leaf and stem cutin deposition. Knocking out any of these genes individually, or as a pair (*gpat4*/*gpat8*), has led to large reductions in Arabidopsis cutin monomer loads, especially in the major monomer octadeca-6,9-diene-1,18-dioate (**1**) (C18:2 DCA). *sn*-2-GPATs play pivotal roles in providing glycerol (**6**) as a structural monomer for cutin through regiospecific *sn*-2 acylation of glycerol-3-phosphate (Yang et al., 2010, 2012). Depending on the monomer composition, putative three dimensional cutin structures could be a linear polyester chain, a dendrimeric structure or a heavily cross-linked domain (Pollard et al., 2008).

Since ω-OHFAs represent less than 20% of total aliphatics of Arabidopsis stem and leaf cutins, and DCAs are the major constituents, Arabidopsis stem and leaf cutins are referred to as DCA-rich cutins thereafter. The reduced compositional complexity of these particular cutins, as well as the availability of mutants that are blocked at different steps of cutin synthesis, offer an opportunity to probe the relationship of cutin aliphatic monomer to glycerol. In this regard, the stoichiometry of DCA-rich cutin monomers allows some inferences to be made about cutin structure. Although glycerol has been detected as an Arabidopsis leaf cutin component (Franke et al., 2005), its quantitative measurement has yet to be made. A major unanswered question is whether glycerol (**6**) is central for cross-linking aliphatics to form cutin structure(s) or whether its main function is as a carrier of fatty acyl groups for cutin assembly (Pollard et al., 2008). To address these issues, a stable isotope dilution GC/MS method was developed for quantitative analysis of glycerol (**6**) with simultaneous determination of aliphatic monomers. In addition, Arabidopsis mutants were utilized to explore the possible structural correlation between glycerol and fatty acid monomers. The molar ratio of glycerol:DCAs was 2:1 in both wild-type stem and leaf cutins. Even when allowing for a modest additional contribution from ω-OHFA, this is a substantially higher glycerol to aliphatic monomer ratio than previously reported for any cutin. Analyzing Arabidopsis mutants defective in genes/enzymes that are essential for DCA-rich cutin accumulation showed that the reduction of glycerol (**6**) content remains proportional to the reduction of DCAs. These findings provide new insights for the role of glycerol (**6**) in DCA-rich cutin structure. Glycerol-DCA-glycerol is proposed as the basic structural motif, while an extensive aliphatic domain, that is, a simple acylglycerol copolymer, is likely not present. A large discrimination between transmethylation rates for acylglycerols versus simple primary and secondary aliphatic esters is also reported, a discrimination that may benefit future cutin monomer analyses.

2. Results

2.1. Overview of analytical methods

For their phytochemical screen Graça et al. prepared leaf and fruit cuticles by digestion with cellulase and pectinase, depolymerized the preparations by NaOMe-catalyzed transmethylation, evaporated the total methanolysate to dryness, and analyzed the silylated monomer products by GC (Graça et al., 2002). 1,12-Dodecanediol was used as an internal standard for glycerol quantification, but as this molecule shares limited structural similarity and physical properties to glycerol (**6**), it is not ideal. Our initial glycerol (**6**) analysis attempts using 1,2-alkanediol as internal standard were less consistent and produced lower glycerol (**6**) levels, suggesting that they could not account for all glycerol (**6**) losses. The low boiling points of compounds such as glyceraldehyde and 1,2-propanediol excluded them as internal standards for glycerol (**6**) quantification (Graça and Pereira, 2000c). In studies with

suberin, the glycerol (**6**) remaining in the aqueous phase after solvent extraction of the depolymerization reaction mixture has sometimes been quantified by enzyme assay (Moire et al., 1999; Schmutz et al., 1993, 1996).

Accurate determination of the glycerol (**6**) to aliphatic molar ratio can provide insights into the role of glycerol (**6**) in cutin structure and define the range of possible polymer linkages. In addition to ensuring complete depolymerization and then quantitative determination of aliphatic and glycerol (**6**) monomers, it is important to know if all glycerol (**6**) released is from cutin rather than from residual membrane lipids or other structures within plant tissues. Optimal conditions for glycerol (**6**) analysis were established and tests for sources of interference or errors that could potentially influence the analysis were run. In the following sections, details of the optimization and validation of our methods are provided. As with earlier work with cutin (Bonaventure et al., 2004), chemical and/or enzymatic preparation of isolated cuticles prior to analysis was considered an opportunity for loss of monomers, particularly if a water-soluble monomer like glycerol was released. Thus experiments were conducted with intact tissues that had been heat/solvent quenched to inactivate lipases and then thoroughly solvent extracted. To quantify glycerol (**6**), the usual organic solvent-aqueous phase partition that follows depolymerization was avoided, while ^{13}C-labeled glycerol (**6**) was used to provide a stable isotope dilution assay of glycerol via GC/MS. This allows for simultaneous quantitative analysis of glycerol (**6**) and aliphatic monomers. This stable isotope dilution method should completely control for all types of loss of glycerol (**6**) after its release during transmethylation. An outline of the overall method is shown in Fig. 2. However, even with optimized protocols, depolymerization of a poorly defined matrix produces results that have some uncertainties in interpretation. Therefore, after completing our analyses on wild type (WT) Arabidopsis, a series of Arabidopsis mutants that are blocked in different steps of cutin monomer synthesis were used as a tool to evaluate the stoichiometric relationship between aliphatic and glycerol (**6**) components. This not only provides confirmation of biosynthesis, but allows a much greater degree of confidence in making structural inferences from the compositional data.

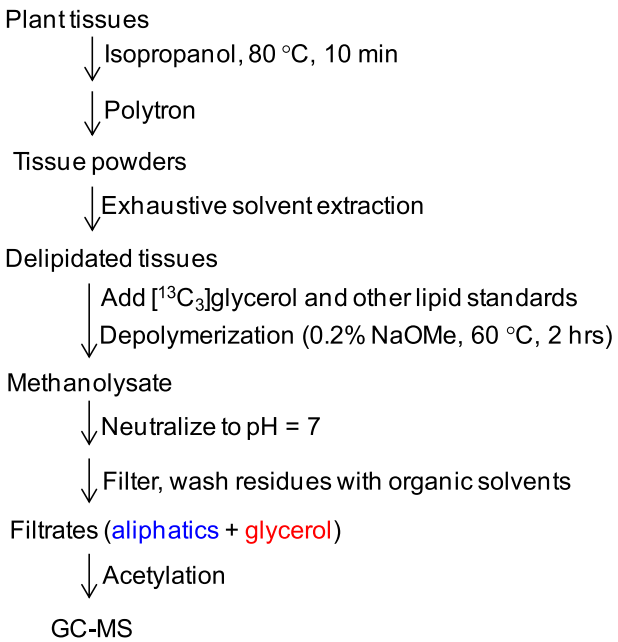

Fig. 2. Flow chart of simultaneous quantitative analysis of glycerol (**6**) and aliphatic monomers (**1**–**5**) from cutin polyester. Fresh plant tissues were heat quenched and ground using a polytron tissue homogenizer before delipidation. [$^{13}C_3$]Glycerol and other lipid standards as described in Experimental were added to thoroughly delipidated dry tissues before transmethylation.

2.2. Isotope dilution analysis by GC/MS provides for accurate quantification of glycerol

Accurate quantification of glycerol (**6**) by GC/MS depends on an efficient derivatization process. In our hands, neither trimethylsilylation nor acetylation gave complete derivatization of glycerol. Since acetyl derivatives are more stable than silylation products and have a longer GC retention time, acetylation was used. Optimal reaction conditions yielded about 70% of the expected glycerol triacetate (triacetin) product in control reactions. The observed difference may result from either the loss of triacetin during sample preparation or incomplete acetylation of the glycerol (**6**). Isotope-dilution mass spectrometry is the preferred internal standard approach to correct for these losses (reviewed in Watson, 1990), and thus to establish an accurate internal standard-based method for glycerol (**6**) quantification. [$^{13}C_3$]Glycerol (**6**) provides a readily available internal standard with the same physiochemical properties of the target analyte (i.e. glycerol (**6**)) and should undergo the same losses during sample preparation and derivatization steps.

For isotope dilution analysis to function, the analyte must have a discrete isotopomer signal. EI-MS spectra of triacetin and [$^{13}C_3$*glyceryl*] triacetin are shown in Fig. 3A. The spectrum of triacetin is very similar to that reported in the NIST Chemistry WebBook (http://webbook.nist.gov/chemistry), while that of [$^{13}C_3$*glyceryl*] triacetin retains the same diagnostic features as reported in a clinical study (Edwards et al., 2012). Important ions for triacetin are shown in Fig. 3B. No parent ion (m/z = 218) was detected, nor was m/z = M-18, also a diagnostic low abundance ion for triacylglycerols. The acetyl cation (m/z = 43) is present as the base peak. Ions at m/z = 103, 115 and 116, and 145 have substantial abundance, and were considered as monitoring ions. However, the ions at m/z = 103 and 145 both have complexity in that each results from two independent modes of fragmentation, only one of which retains glycerol carbon backbone atoms. This conclusion occurred because [$^{13}C_3$*glyceryl*] triacetin gives pairs of ions at m/z = 103 and 105, and at m/z = 145 and 147. By contrast, the radical cation at m/z = 116 is derived formally by the loss of acetic anhydride (or acetic acid plus ketene) from the molecular ion and thus retains all glycerol (**6**) carbon atoms. Further loss of one H atom (or loss of acetic acid plus an acetylium radical from the molecular ion) produces the cation at m/z = 115. Thus [$^{13}C_3$*glyceryl*] triacetin produces ions at m/z 118 and 119, relative to 115 and 116 in unlabeled triacetin. Based on this analysis of fragmentation, ion pairs 115/116 and 118/119 were chosen to monitor triacetin and [$^{13}C_3$*glyceryl*] triacetin, respectively. To increase the MS signal and assay sensitivity, the GC/MS instrument was operated in the SIM mode with four ions (115, 116, 118 and 119) across the triacetin peak. Ratios of either 115/118 or 116/119 ion pair can be used for glycerol quantification.

To establish that the ratios of ion currents are linear functions of the molar ratio of analytes, a series of standard mixtures were prepared with a fixed amount of [$^{13}C_3$]glycerol and varying amount of glycerol to give a range of molar ratios of glycerol to [$^{13}C_3$]glycerol of 0–2. After acetylation and analysis by GC/MS, the ratio of ions 115 and 118, and ions 116 and 119 were calculated and plotted as a function of the known molar ratio of glycerol to [$^{13}C_3$]glycerol (Fig. 3C). Assessment of these calibration curves indicated no significant difference in using either pair, as $r^2 > 0.99$ in each case. Ion pair 116/119 was routinely used for our glycerol quantification in this study.

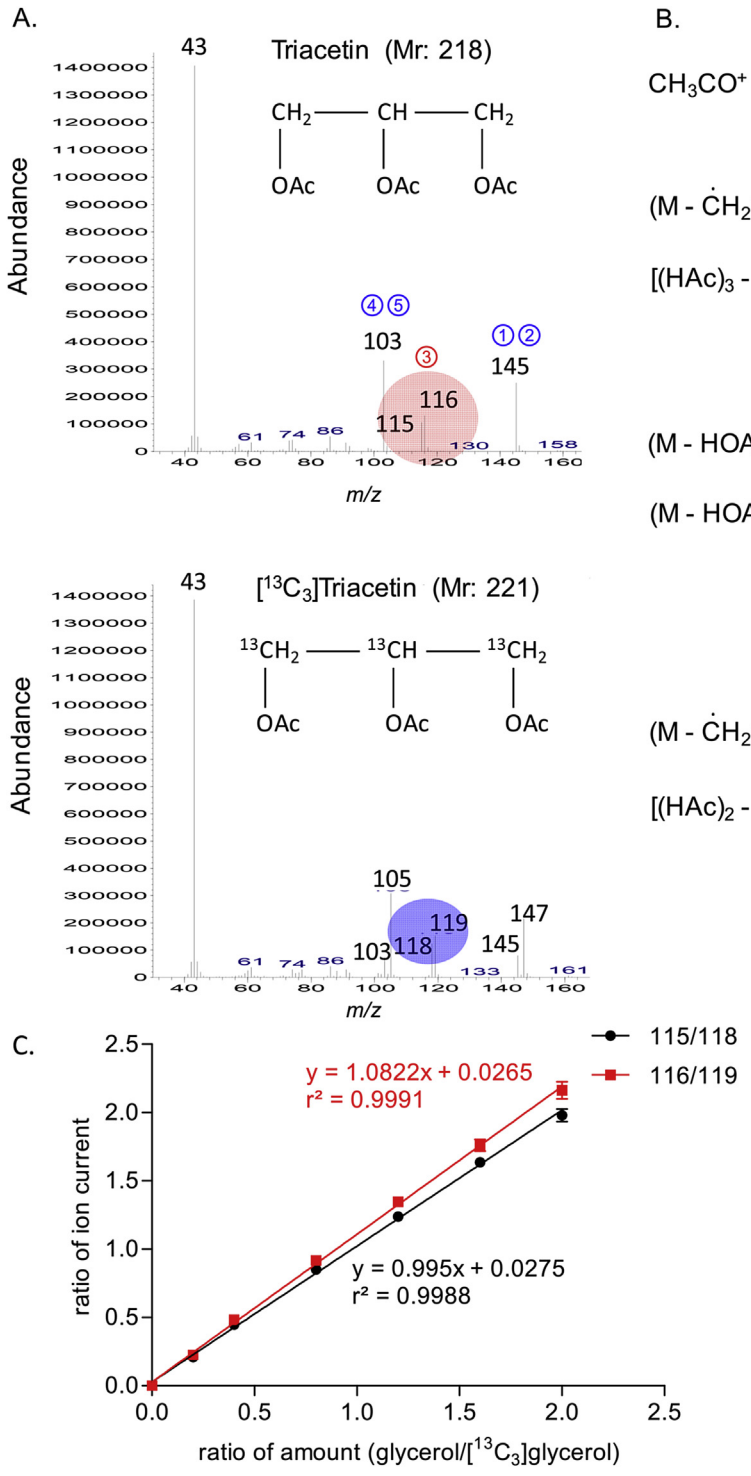

Fig. 3. Quantitative analysis of glycerol (**6**) by stable isotope dilution assay. (A) EI mass spectra of triacetin and [$^{13}C_3$*glyceryl*]triacetin. (B) Deduced fragmentation patterns for triacetin. The fragmentations are rationalizations, not experimentally defined mechanisms. For example, m/z = 116 may be written as $(M\text{-}Ac_2O)^{+\bullet}$ or as $(M\text{-}HOAc\text{-}CH_2CO)^{+\bullet}$. Further evidence that the number of glycerol (**6**) and acyl group carbon atoms in triacetin fragments is correct comes from inspection of the mass spectra of higher TAG homologues (tripropanoin and tributyrin, NIST Chemistry WebBook, http://webbook.nist.gov/chemistry). In particular, the overlapping fragmentations for m/z = 103 and 145 ions separate by the expected m/z values in these homologues. (C). Calibration plots for ratios of ion current at the indicated masses versus ratios of molar amounts of triacetin and [$^{13}C_3$]triacetin. Under the SIM mode, the dwell time is 200 ms. Values represent mean ± SD (n = 3).

2.3. Optimization of the methanolysis reaction and monomer recovery

Analysis of cutin monomer composition requires depolymerization to cleave the ester bonds within the polymer. Since acid-catalyst transmethylation, using strongly dehydrating acids like sulfuric acid, will likely reduce glycerol (**6**) yields by formation of its dehydration product, acrolein, the depolymerization process is better accomplished by base-catalyzed transesterification, typically with NaOMe at 4–6% w/v in methanol (Bonaventure et al., 2004;

Molina et al., 2006). Following transmethylation, addition of organic solvent and phase partition is routinely used to recover the aliphatic monomers (examples are **1**–**5**) for further derivatization and analysis by GC or GC/MS. Glycerol (**6**) remains in the aqueous phase and can be assayed enzymatically. Isotope dilution analysis precludes enzyme assay. In the current analysis, the NaOMe concentration for transmethylation was reduced to levels used by Graça and Pereira (2000b) and Graça et al. (2002), to avoid the subsequent phase partition step, and to facilitate the synchronous analysis of both glycerol (**6**) and aliphatic monomers.

To set up the method outlined in Fig. 2, aliphatic monomer recovery was first assessed as a function of catalyst concentration. Quantification of the mass of aliphatic monomers was based on their total ion currents measured against methyl heptadecanoate as an internal standard. In addition, ω-pentadecalactone was used to monitor for complete transmethylation of primary esters and hydroxy group derivatization (Bonaventure et al., 2004; Molina et al., 2006). Since the major aliphatic component of Arabidopsis stem and leaf cutin is C18:2 DCA (**1**), methyl *cis,cis*-11,14-eicosadienoate (i.e. C20:2 fatty acid methyl ester (FAME)) was included as another internal standard to monitor the potential oxidation of C18:2 DCA (**1**) during the analysis. Delipidated Arabidopsis stem tissues were depolymerized under the standard conditions (60 °C for 2 h in methanolic NaOMe containing 15% by volume MeOAc) at 0.05–6% w/v NaOMe concentrations. After neutralization with dilute aqueous HCl, the aliphatic monomers were recovered by extraction with CH_2Cl_2. The lowest concentration of NaOMe that resulted in high recovery of aliphatic monomers was 0.1% w/v (Supplemental Fig. S1A). However, at this concentration, incomplete ω-pentadecalactone transmethylation was observed (Supplemental Fig. S1B). Similar results were obtained with Arabidopsis leaf cutin analysis (data not shown). Since 0.2% w/v NaOMe was the lowest concentration that permitted both good recovery of total DCAs and complete derivatization of ω-pentadecalactone, it was used as the standard catalyst concentration for Arabidopsis stem and leaf cutin transmethylation. The residue recovered after evaporation of the filtered methanolysate was then acetylated under conditions described in Experimental.

2.4. Controls and validations for the protocol

With transmethylation and glycerol analysis methodology defined, a series of validation and control experiments were performed. The first of these analyzed mixtures of diacylglycerols (DAGs) and triacylglycerol (TAGs) along with a FAME internal standard. [$^{13}C_3$]Glycerol was added to each mixture as an internal standard for glycerol (**6**) quantification. Aliquots of these mixtures were transmethylated, acetylated and analyzed by GC/MS. Details of the test mixtures and the results are given in Supplemental Fig. S2. The measured molar ratios of FAME to glycerol released from DAG and TAG species in all three mixtures were similar to the calculated theoretical values confirming that the overall method is suitable for accurate quantification of both glycerol (**6**) and aliphatic monomer components.

The rates of release of glycerol (**6**) and DCAs from the polymer matrix may not be identical. Therefore it is important to assess that the transmethylation reaction has gone to completion for both substrates. The releases of DCAs, ω-OHFAs and glycerol (**6**) from WT Arabidopsis delipidated leaf residues were measured for 0.5, 2, 17 and 24 h transmethylation reactions. All monomer concentrations and thus their ratios remained constant throughout the time course (Fig. 4), indicating the reaction reached its end point as early as 0.5 h and that monomer degradation did not occur over extended reaction periods. DCAs were the major fatty acid species released from Arabidopsis leaf cutin, representing ≥95% of the released

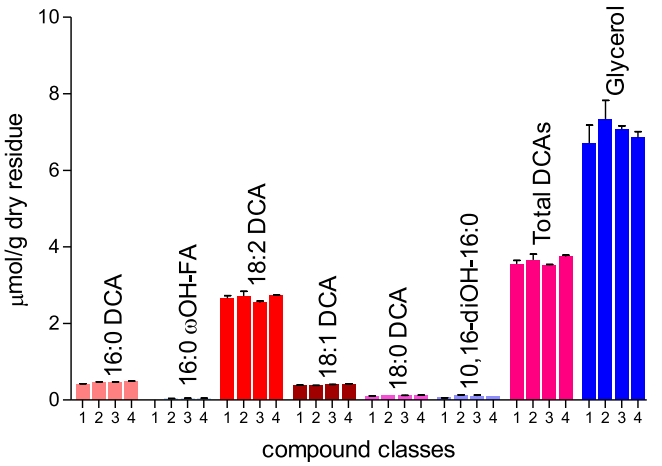

Fig. 4. Time course of monomer release from WT Arabidopsis leaf cutin by 0.2% NaOMe-catalyzed transmethylation. 1: 0.5 h; 2: 2 h; 3: 17 h; 4: 24 h. The values represent mean ± range (n = 2).

aliphatics. It was concluded that the 2-h-reaction time for transesterification was sufficient for releasing both DCAs and glycerol (**6**) from Arabidopsis stem and leaf cutin.

Glycerol release does not necessarily imply either partial or complete association with the cutin matrix. Free glycerol (**6**) has been reported in leaves (Gerber et al., 1988). Such glycerol (**6**) should be readily extracted by polar solvents during tissue quenching and delipidation. To confirm this, glycerol (**6**) analyses were run for delipidated Arabidopsis leaf residues with transmethylation at 60 °C, at room temperature, and without transmethylation catalyst at 60 °C. Glycerol (**6**) concentrations were 7.0 ± 0.45, 6.5 ± 0.17 and <0.1 μmol/g dry weight residues (n = 3), respectively. Since glycerol (**6**) could barely be detected (90-fold lower) when the transmethylation catalyst NaOMe was omitted from the analysis, free glycerol (**6**) is a negligible contaminant in Arabidopsis cutin preparations.

One potential source of glycerol (**6**) is residual membrane glycerolipid. Although preparation of Arabidopsis leaf and stem tissues for cutin analysis involves extensive solvent extraction to remove membrane lipids, glycerolipids in leaf and stem tissues are present at much higher levels than cutin. The total membrane glycerolipid content of Arabidopsis leaves is approximately 165 μmol/g dry weight (63 and 102 μmol/g dry weight respectively for phospholipids and glycolipids) (Welti et al., 2002). This is more than 48-fold higher than the total amount of ω-oxidized aliphatic monomer content of 7-week-old leaf cutin in our current analysis (3.4 ± 0.3 μmol/g dry weight). To test for glycerol release from polar lipids, a series of standards were transmethylated and analyzed (Fig. 5). For phosphatidylcholine, mono- and di-galactosyldiacylglycerol standards 91%, 94% and 107% of the theoretical yields of FAME were recovered, but in all cases the release of free glycerol (**6**) was <2% of the theoretical value. Furthermore, the release of free glycerol (**6**) from glycerol-3-phosphate was essentially undetectable (<1%), confirming the result from phosphatidylcholine. Thus it appears that the glyceryl-phosphate ester bonds of phospholipids and the glyceryl-ketal bonds of galactolipids will not be cleaved. Therefore, glycerol (**6**) release from residual membrane lipids will be negligible under the cutin depolymerization conditions. The fact that glyceryl-phosphate ester bonds are recalcitrant to cleavage by transmethylation removes a concern that glycosylphosphatidylinositol-anchored proteins could contribute to the free glycerol signal. These plasma membrane proteins are unlikely to be extracted by organic solvents during

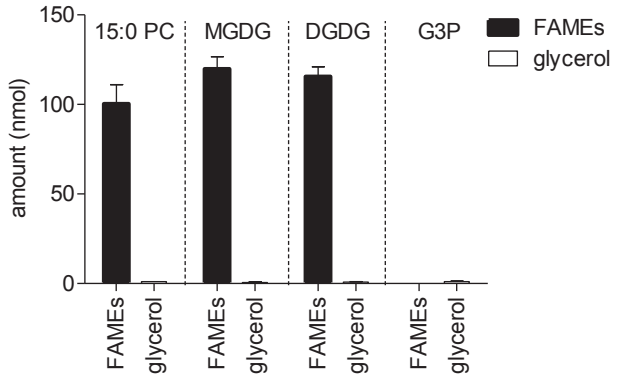

Fig. 5. Glycerol (**6**) from membrane lipid standards and glycerol-3-phosphate (G3P) is not released during transmethylation. Known amounts of phospholipid standard dipentadecanoyl phosphatidylcholine (PC, 57 nmol), glycolipid standards monogalactosyldiacylglycerol (MGDG, 64.5 nmol) and digalactosyldiacylglycerol (DGDG, 53 nmol), and G3P (120 nmol) were transmethylated with 0.2% NaOMe at 60 °C for 2 h. The values represent mean ± SD (n = 3).

delipidation process and could be present at levels of the order of 0.1 μmol glycerol (**6**) per gram dry residue. In our current analysis, glycerol levels in Arabidopsis stem and leaf cutin preparations were 3.3 and 6.4 μmol/g dry residue, respectively (Fig. 6).

DCAs released from Arabidopsis cutin polyester by NaOMe-catalyzed transmethylation are dimethyl diesters (Bonaventure et al., 2004; Franke et al., 2005), indicating that both ends of the molecule are esterified in vivo. However, monoesters are possible if some of the DCA is linked into cutin through only one carboxylate group. To explicitly test for and quantify this possibility, a mixture of dimethyl octadec-9-*cis*-en-1,18-dioate and its free acid (C18:1 DCA, **2**) was heated at 100 °C overnight to randomize esterification. It was then silylated to generate C18:1 DCA (**2**) monomethyl-monoTMSi-diester standard. Its GC retention time and identity were confirmed by GC/MS (Supplemental Fig. S3). Since acetylation would not allow us to monitor this possible hydrolysis, trimethylsilylation was employed after transesterification. TMSi-derivatized methanolysates of both Arabidopsis leaf and stem cutins were examined. Based on comparison with the retention time and mass spectra of the above standard, as well as C18:1 DCA (**2**) bis-TMSi ester, neither mono- nor bis-TMSi C18:2 DCA (**1**) could be detected by GC/MS. This excludes both the possibility of release of the half ester during cutin depolymerization and of subsequent hydrolysis of C18:2 DCA (**1**) dimethyl diesters later in the analysis.

Results presented above (section 2.3 and 2.4) indicated that a 0.2% NaOMe transmethylation concentration gave complete recovery of DCAs and glycerol (**6**) but did not release free glycerol from phospholipids nor galactolipids. To further test the effect of NaOMe concentration on depolymerization efficiency for cutin polymer, Arabidopsis WT leaf cutin was depolymerized with NaOMe at the concentrations of 0.2%, 6% or 12%, respectively. The reaction mixtures were acidified and extracted with CH_2Cl_2 to recover DCAs and other aliphatics for GC/MS. Under all the concentrations tested, the recovery of DCAs remained at the same level (Supplemental Fig. S4). However, there was a noticeable difference in the recovery of ω-OHFAs at different NaOMe concentrations. In particular, the amount of 10,16-dihydroxypalmitate (**5**) recovered after transmethylation in 6% or 12% NaOMe was about four fold higher than that released by 0.2% NaOMe. This exception may arise from its physical accessibility within the insoluble cell wall matrix or differential chemical reactivity of carboxylate ester linkages. Nevertheless, 0.2% of NaOMe was suitable to release all DCAs, the major class of aliphatics in Arabidopsis leaf cutin.

Because of the differential release of monomers with NaOMe catalyst concentration, a series of lipid ester substrates were transmethylated using 0.2% NaOMe in methanol plus methyl acetate, with the reactions being carried out at room temperature to facilitate kinetic analysis (Supplemental Fig. S5). The controls above show that even at room temperature, the release of glycerol from cutin by transmethylation is essentially complete. Low lipid substrate concentrations (0.7–2.1 mM) were used to ensure substrate solubility in the reaction medium. The transmethylation rate differences between acylglycerols and isolated primary and secondary ester bonds for these hydrophobic substrates were substantial. The reaction half-lives, calculated from the first order rate constants, exhibited ratios of 1:25:700 for triolein, oleyl oleate and 12-palmitoyl-ricinoleate (**7**), respectively. Clearly the potential exists for differential ester bond reactivity to play a very significant role in the differential release of cutin monomers.

2.5. The molar ratio of glycerol to DCAs in Arabidopsis leaf and stem cutin and Brassica napus leaf cutin is approximately 2:1

The presence of glycerol (**6**) has been detected, but not quantified in isolated Arabidopsis leaf cuticles (Franke et al., 2005). Here, based on the above established isotope dilution GC/MS method, the glycerol contents in both Arabidopsis stem and leaf cutin preparations were determined. The glycerol (**6**) amounts in stem and leaf cutin were 35 and 36% (w/w) of the identified monomers, while the total amounts of DCAs were 61 and 58% (w/w) of the methanolysates, respectively. To evaluate the role of glycerol (**6**) in DCA-rich cutin structure, the molar ratios of glycerol to DCAs in Arabidopsis WT stem cutin and leaf cutin were calculated. In Arabidopsis stem cutin preparations, the amounts of glycerol (**6**) and total DCAs were 3.3 and 1.7 μmol/g dry residue, respectively. In Arabidopsis leaf cutin, the amounts of glycerol (**6**) and DCAs were 6.4 and 3.1 μmol/g dry residue (Fig. 6). The amounts of total ω-OHFAs (including 10,16-dihydroxypalmitate (**5**)) in stem and leaf cutin were 0.11 and 0.34 μmol/g dry weight. Thus, in both stem and leaf cutin, the molar ratio of glycerol (**6**) to DCAs was approximately 2:1. As a further examination of glycerol (**6**) content in DCA-rich cutin, *B. napus* leaves from both 18-day-old and 60-day-old plants were analyzed. The 2:1 ratio of glycerol (**6**) to DCAs was also observed for these tissues using the 0.2% NaOMe transmethylation protocol (Supplemental Fig. S6).

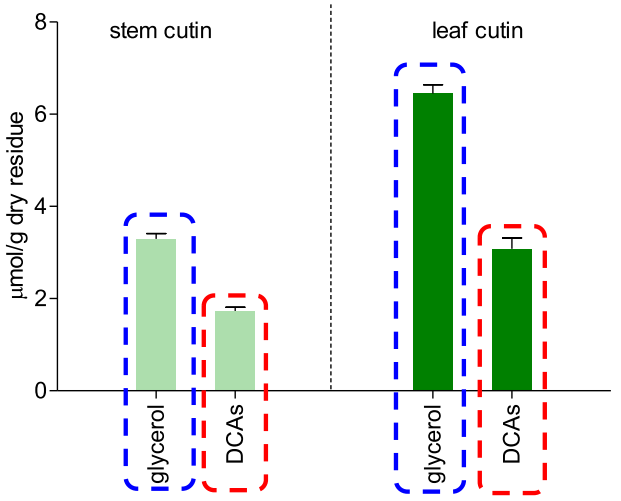

Fig. 6. Molar amounts of glycerol (**6**) and DCAs (**1**–**3**) in Arabidopsis WT stem and leaf cutins. Three different batches of plant tissues were used for the analysis with duplicates for each batch. Values represent mean ± SD (n = 6).

2.6. Glycerol and DCAs are proportionally reduced in leaf and stem in Arabidopsis cutin mutants

Quantitative analysis of fatty acyl monomers (such as **1**–**5**) and glycerol (**6**) in Arabidopsis mutants with lesions in cutin biosynthesis is required and can provide complementary validation of our methodology. Three types of mutants, in reactions that are essential for Arabidopsis cutin biosynthesis, were previously identified. 1) GPAT4 and GPAT8 are glycerol-3-phosphate 2-*O* acyltransferases that catalyze the first step in leaf and stem cutin acylglycerol assembly (Yang et al., 2010, 2012). The double-knockout *gpat4*/*gpat8* has an overall 60–70% reduction of aliphatic monomers in stem and leaf cutin, and the most pronounced decrease occurs in its major monomer, C18:2 DCA (**1**) (Li et al., 2007a). 2) *ATT1* encodes cytochrome P450 CYP86A2, which is required for oxidation of the omega-terminal carbon of aliphatic chains in leaf and stem cutin biosynthesis. Mutation of this gene leads to reductions of approximately 70% of C18:2 DCA (**1**) in leaf cutin and approximately 50% in stem cutin, respectively (Molina et al., 2008). 3) The acyl-CoA synthetase mutant *lacs2-3* has 80% and 70% reductions in DCA in Arabidopsis leaf and stem cutin, respectively (Bessire et al., 2007; Lü et al., 2009). Since Arabidopsis *gpat4*/*gpat8*, *att1-2* and *lacs2-3* mutants all have significant reduction in DCAs, they were chosen for further glycerol analysis.

Compared with WT cutin, the reductions in C16:0 (**3**), C18:1(**2**) and C18:2 (**1**) and other minor DCAs in stem and leaf cutins of *gpat4*/*gpat8*, *att1-2* and *lacs2-3* mutants were confirmed to be very similar to previous reports by our current methodology (Supplemental Fig. S7). Glycerol (**6**) analysis indicated its fractional reduction in each mutant corresponding to the fractional reduction of total DCAs in both stem and leaf cutin. In the *gpat4*/*gpat8* double knockout mutant, the molar reductions of glycerol (**6**) and DCAs in stem cutin were 88% and 87%, respectively. There was a 49% reduction of glycerol (**6**) and a 51% reduction of total DCAs in stem cutin analysis for the *att1-2* mutant. Analysis of *lacs2-3* indicated molar reductions of glycerol and DCAs of 57% and 45%, respectively (Fig. 7A). Leaf cutin analysis of the mutants yielded similar reduction patterns for both glycerol (**6**) and DCAs (Fig. 7B). Thus the glycerol:DCA molar ratio of 2:1 seen in WT Arabidopsis is reduced at a constant 2:1 ratio in three distinct mutants. This implies a direct association and structural motif as will be discussed below. Furthermore, it is difficult to explain any glycerol (**6**) from our analysis as originating from other than cutin. This confirms the expectation from the controls that glycerol "contamination" from other sources will be minimal.

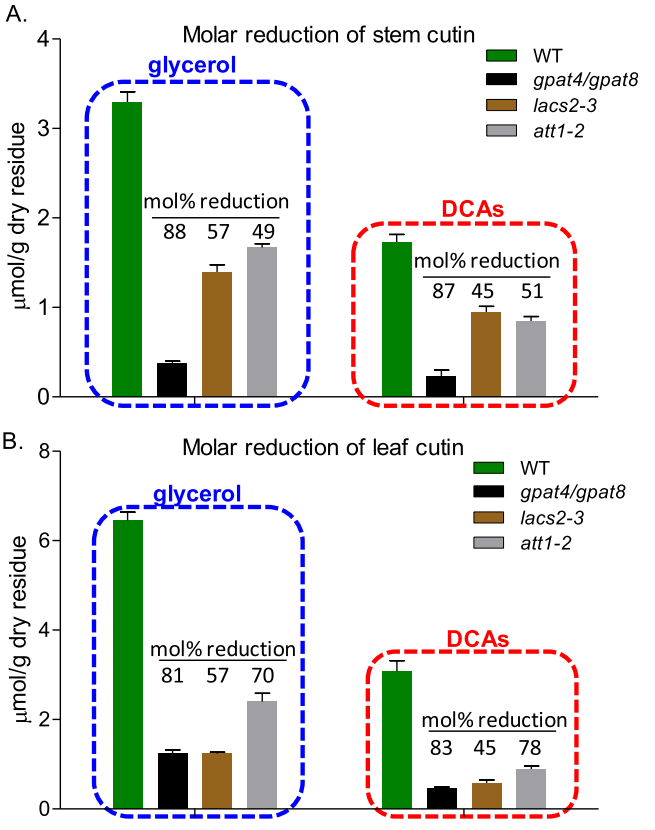

Fig. 7. Mole percentage reductions of glycerol (**6**) and DCAs (**1**–**3**) in (A) stem cutin and (B) leaf cutin of Arabidopsis *gpat4*/*gpat8*, *lacs2-3* and *att1-2* mutants. Three different batches of plant tissues were used for the analysis with duplicates for each batch. Values represent mean ± SD (n = 6).

3. Discussion

3.1. Inferences from controls for the measurement of glycerol and DCA monomers in cutin

Measurement of the molar ratio of glycerol (**6**) to aliphatic monomers (such as **1**–**5**) in cutin can contribute insights into its assembly and three-dimensional structure. However, such a stoichiometric analysis presents difficulties because cutin is an insoluble polymer, or more likely a heteropolymer with linkages to the plant cell wall. As reviewed in the Introduction, bonding connectivity between monomers can be demonstrated by partial hydrolysis (or transmethylation) and analysis of the resulting oligomers by GC/MS or ESI-MS. However, partial depolymerization usually provides only a small fraction (~5%) of the material and a complete accounting of the ester linkages is not possible. MAS-NMR methods have been applied to cutin preparations and give averaged compositional data, but full interpretation of polymer structure remains elusive. If cutin is really a cell wall heteropolymer, preparation of enriched fractions by chemical or enzymatic treatments may perturb structure.

This problem was tackled using Arabidopsis stem and leaf tissues, as they provide relatively simple aliphatic monomer compositions, dominated by DCAs, and because mutants are available for several biosynthetic genes. These mutants give strong chemical and biological phenotypes. However, the first requirement is a robust and fully controlled glycerol analysis to complement the aliphatic monomer analysis. An isotope-dilution method using [$^{13}C_3$]glycerol was developed to measure glycerol concurrently with aliphatic monomers. [$^{13}C_3$]Glycerol is added to the transmethylation reaction and appears to be a better standard than chemically less-identical structures.

The mild NaOMe-catalyzed transmethylation conditions employed allow accurate determination of FAME to glycerol (**6**) ratios in TAG and DAG standard mixtures (Supplemental Fig. S2). The conditions permit complete cleavage of the hydrophobic primary ester linkage in pentadecalactone internal standard (Supplemental Fig. S1). Time courses for transmethylation of Arabidopsis leaf tissues show complete reaction for the release of glycerol (**6**) and aliphatic (DCA) monomers (Fig. 4). The plant tissues have been exhaustively delipidated and are expected to have negligible amounts of residual soluble neutral and polar lipids. Controls with lipid standards show that under the transmethylation conditions, glycerol (**6**) will not be released from phospholipids or galactolipids (Fig. 5). Although transmethylation will cleave fatty acids from these polar lipid standards, phosphodiester bonds are clearly resistant to reaction. Likewise, the galactopyranosyl(1′-3)-*sn*-glycerol linkage remains intact. This is

expected since ketals are resistant to base hydrolysis. An important inference from these controls (Fig. 5) is that it is more certain that glycerol (**6**) is attached to the cell wall matrix by carboxylate ester bonds. It will not come from residual lipids or non-extracted species like glycosylphosphatidylinositol-anchored proteins.

Unesterified fatty acids are common constituents of cuticular waxes (Kunst and Samuels, 2003) and suberin-associated waxes (Espelie et al., 1980; Li et al., 2007b). Their occurrence raises the possibility that DCAs or a fraction thereof may have one esterified and one free carboxylate group in vivo. If such were the case, base-catalyzed transmethylation and subsequent acetylation would produce the mono-methyl ester of DCA. However, no DCA mixed ester/acid products were observed when Arabidopsis stem and leaf transmethylation products were analyzed by GC/MS after silylation rather than the standard acetylation. Thus both carboxylic acid groups in cutin DCAs are esterified in vivo.

3.2. Proposing "glycerol-DCA-glycerol" as a dominant motif in DCA-rich cutin

A correlation between glycerol (**6**) and DCA levels has been noted for a variety of suberins. The molar glycerol to DCA ratios for each suberin type varied between 1:1.3 and 1:3.3 (Moire et al., 1999). Most cutins are composed mainly of C16 and/or C18 ω-OHFA aliphatic monomers. These monomers often have mid-chain functionality such as hydroxy, keto or epoxy groups. DCA monomers constitute much lower fraction of total aliphatic monomers in cutin than in suberin. Thus the identification of high levels of DCAs (40–60 mol %), and particularly C18:2 DCA (**1**), in Arabidopsis leaf and stem cutins was unexpected (Bonaventure et al., 2004; Franke et al., 2005). The presence of a polyunsaturated aliphatic monomer was also unusual. There were also significant amounts of C18:1 DCA (**2**) and C16:0 DCA (**3**). In a study of the composition of ω-OHFA-rich cutin preparations from seven plant species, glycerol (**6**) levels of 1.1–13.8% of total aliphatic monomers (w/w) were reported (Graça et al., 2002). This corresponds to a molar ratio of glycerol (**6**) to aliphatic monomer of approximately 1:2 to 1:36. To minimize the error in the analysis, the molar ratio of glycerol (**6**) to total DCAs was analyzed with duplicates on three different batches of plant tissues, for a total of 6 analyses for stem and 6 for leaf. This allowed us to provide confidence levels. The molar ratios of glycerol (**6**) to DCAs in Arabidopsis stem and leaf tissues were 1.91 ± 0.11 SD and 2.09 ± 0.14 SD, respectively. Thus Arabidopsis leaf and stem cutin reported here, with an inverted ratio approaching 2:1, was unexpected and implied an unusual cutin structure.

Controls show that release of glycerol (**6**) and DCA monomers (**1**–**3**) by transmethylation of Arabidopsis leaf and stem tissues is complete, that no glycerol (**6**) originates from residual phospholipids or glycolipids in the delipidated tissue, that glycerol (**6**) is most likely esterified via only carboxylate ester bonds, and that both carboxylate groups of DCAs are esterified in vivo. Analyses of Arabidopsis WT stem and leaf (Fig. 6) and *B. napus* leaf (Supplemental Fig. S6) tissues produce molar ratios of glycerol (**6**) to DCAs very close to 2:1. The simplest explanation for this is that the basic structural motif for this type of cutin is "glycerol-DCA-glycerol". Such an oligomer has been identified by partial depolymerization of suberin (Graça and Pereira, 2000b), giving credence to the possibility for a similar structural motif in cutin. However, the stoichiometric 2:1 ratio could be fortuitous, with glycerol (**6**) from another, unrelated structure adding to the glycerol (**6**) released by cutin depolymerization. Analysis of the Arabidopsis mutant lines suggests this possibility is very unlikely because the loss of glycerol (**6**) and of DCA in each of the mutants also occurs at a 2:1 stoichiometric ratio (Fig. 7 and Supplemental Fig. S7). This is true for each of the three genes/enzymes in the pathway for which we have mutants, namely the acyl-CoA synthetase (LACS2), the CYP86A2 P450 oxidase (ATT1), and the pair of redundant *sn*-2 acyltransferases (GPAT 4/8). Thus 2:1 glycerol:DCA appears to be a biosynthetic unit.

The glycerol-DCA-glycerol motif is shown in the schematic in Fig. 8. Previous work with the GPAT4/8 pair demonstrated that this enzyme acylates glycerol-3-phosphate at the *sn*-2 position (Yang et al., 2010, 2012). These enzymes also have a functional phosphatase domain, such that the end product is not *sn*-2-acyl lysophosphatidic acid, but rather *sn*-2-monoacylglycerol. There are no reports of phosphorylation of cutin and suberin. It is therefore likely that dephosphorylation of acylglycerol monomers is complete prior to export or deposition. Glycerol (**6**) contained in any residual phosphorylated monomers would not be detected in our assay, such that the true glycerol:DCA ratio would be underestimated. The *sn*-2 stereochemistry is shown in Fig. 8, but it should be kept in mind that acyl migration or extracellular isomerases (lipases) may produce DCA esterified to the primary position of glycerol in vivo. Evidence for this type of isomerization comes from ectopic expression of the related GPAT5, a suberin biosynthesis enzyme, in Arabidopsis stems, where both α- and β-monoacylglycerols were identified as cuticular wax product (Li et al., 2007b). The partial hydrolysis studies where glycerol-DCA and glycerol-DCA-glycerol oligomers are released cannot predict stereochemistry because the reaction conditions allow acyl migration (Graça and Pereira, 2000b). The exact sequence of glyceryl-DCA biosynthesis in vivo is not entirely clear (Pollard et al., 2008). Specificity towards ω-oxidized acyl-CoA substrates (Yang et al., 2012) suggests ω-oxidation before acyl transfer, but whether the P450 is the only oxidase and whether it acts on free fatty acid or acyl-CoA remains uncertain. The glycerol-DCA-glycerol motif indicates that further studies on *sn*-2-GPAT specificity are warranted.

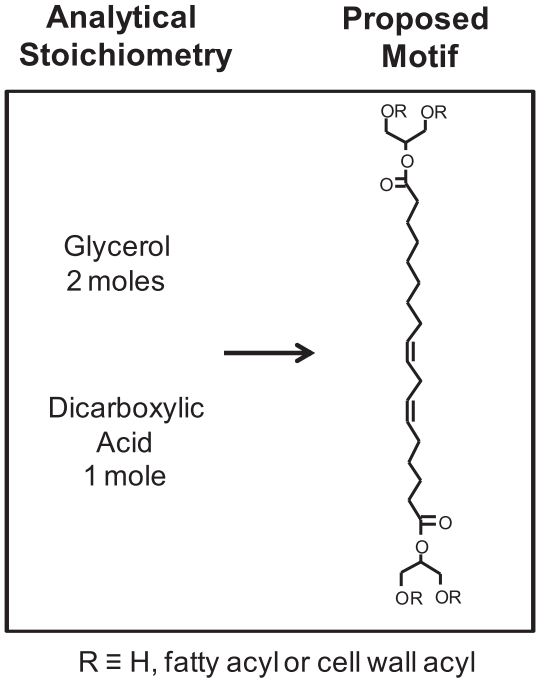

Fig. 8. Putative dominant structural motif of DCA-rich cutin. The 2:1 molar ratio of glycerol (**6**) to DCAs (**1**–**3**) is inconsistent with the existence of an extended poly(-acylglycerol) domain. Instead, "glycerol-DCA-glycerol" is the dominant motif. Of the four remaining glycerol hydroxyl groups at least one, but possibly up to four ester linkages could anchor the glycerol-DCA-glycerol motif to the primary cell wall (for example, via the galacturonic acid residues in pectin). However, some glycerol hydroxyl groups may be free (R≡H) or esterified to other fatty acids.

Previous work with recombinant *sn*-2-GPATs did not contain specific LACS enzymes or cofactors that might lead to a second round of glycerol acylation on DCA substrate.

There are some caveats to proposing glycerol-DCA-glycerol as a dominant motif. First, the possibility remains that transesterification of glyceryl entities could occur (partially or completely) on assembly of glycerol-DCA-glycerol to give glycerol (**6**) and DCA each independently attached to the cell wall matrix. A structural analogy for this occurs for pectin where homogalacturonan is methyl-esterified at *O*-6 and acetylated at *O*-2 and *O*-3 (Atmodjo et al., 2013). Secondly, although the stoichiometric ratio of glycerol (**6**) to DCA is very close to 2:1, as the molar amount of additional monomer fatty acids increases then there is an increasing opportunity for deviation from the motif. This second option is described below.

3.3. Implications, variations and limitations for a glycerol-DCA-glycerol motif in cutin

Considering glycerol and DCA to alone provide the dominant stoichiometric components of cutin, a cutin polymer with a 1:1 molar ratio would have to be a linear polymer (Supplemental Fig. S8A), whereas a 2:3 molar ratio would produce a fully esterified aliphatic structural domain that could be randomly cross linked (Pollard et al., 2008) (Supplemental Fig. S8B) or be an ordered linear variant (Supplemental Fig. S8C). Intermediate ratios could produce polyester domains with a dendrimeric structure (Pollard et al., 2008). However, a 2:1 ratio precludes a simple acylglycerol copolymer. Extended aliphatic polyester domains are commonly presented as cutin structures in the literature. A corollary of the glycerol-DCA-glycerol motif is that in order to be insoluble at least one of the glycerols must be linked to the cell wall. Thus cutin would be better described as a heteropolymer rather than a lipid polymer. Furthermore, given the potential symmetry of glycerol-DCA-glycerol, it is possible that both glycerol groups could be linked to the cell wall matrix. Moreover, if the *sn*-2 position is retained as the site of DCA acylation, both primary hydroxyls are equivalent and may be available for linkage to the cell wall. Thus 1 to 4 ester linkages to the cell wall are possible per glycerol-DCA-glycerol unit. As the glycerol (**6**) is almost certainly linked via a carboxylate ester, uronic acid residues in cell wall polysaccharides would be strong candidates to provide the anchoring linkages. Aspartate or glutamate residues in cell wall proteins could also be considered. Pectins are rich in galacturonic acids and would be found in the upper part of the cell wall where cutin is located (Jeffree, 2006). Also, some cutins have a lamellar appearance by electron microscopy (Jeffree, 2006), observations consistent with heteropolymeric structure. As DCA in Arabidopsis cutin survives the isolation of the cuticle by pectinase plus cellulase treatment (Franke et al., 2005), proposing a glycerol-DCA-glycerol attachment to pectin implies acyl-glyceryl-pectin is resistant to pectinases. How would additional acyl monomers change this hypothesis? Such components may be unseen, or observed (Supplemental Fig. S4). Cutin analysis provides composition for depolymerized monomers. Residual aliphatic monomers remaining covalently linked to the cell wall after depolymerization are collectively called cutan, can vary substantially, and will confound any stoichiometric accounting. Such monomers are proposed to be linked to each other or the cell wall through either C-C or C-O-C bonds, which are not cleaved by the standard depolymerization methods that cleave ester bonds. Given that acylglycerol transmethylation is a facile reaction (Supplemental Fig. S5), glycerol (**6**) release and recovery from cutan should not be impaired. Cutan analysis has not been reported for any Arabidopsis tissue. In *Clivia* and *Agave* species, it is shown that cutin deposition precedes cutan formation, but cutan levels can rise to represent >50% of cuticle dry weight in planta (Riederer and Schonherr, 1988; Schmidt and Schonherr, 1982; Villena et al., 1999). In Arabidopsis, the total aliphatic monomers per surface area decreased approximately 2 fold from upper sections of stems compared to lower, more mature sections (Suh et al., 2005). Such a decrease could reflect lower recovery of aliphatic monomers that are linked by ether bonds formed via peroxidation of unsaturated monomers (Lequeu et al., 2003). If cutan is a major component of the Arabidopsis cuticle, such DCAs or other monomers would either not be released by the transesterification procedure or produce higher MW adducts not detected by GC/MS, resulting in lower ratios of glycerol to acyl monomers of mature Arabidopsis stem and leaf tissues than we currently observe. These altered ratios could allow esterified aliphatic structural domains as described in the previous paragraph.

The release of aliphatic ω-oxidized monomers is clearly dependent on catalyst concentration (Supplemental Fig. S4). At 0.2% NaOMe, all DCAs are released, but only 60% of ω-OHFA and 25% of 10,16-dihydroxypalmitate (**5**). Thus the mild conditions significantly underestimate certain monomers. Total ω-OHFA (including 10,16-dihydroxypalmitate (**5**)) rises from 14 mol % of total aliphatic monomers at 0.2% NaOMe to 28 mol % (includes 17% 10,16-dihydroxypalmitate) at 6–12% NaOMe. It is well known that base-catalyzed transmethylation proceeds more rapidly for acylglycerols compared to wax and sterol esters (Christie, 1993). However, quantitative comparisons appear lacking. The large transmethylation rate differences between acylglycerols and isolated primary and secondary ester bonds for soluble lipid substrates were unexpected (Supplement Fig. S5). The half-life ratios of 1:25:700 for triolein, oleyl oleate and 12-palmitoyl-ricinoleate (**7**) transmethylation, respectively, suggest that significant rate differences will exist for the cleavage of cutin ester linkages. These are likely be modulated by the rate of penetration of the reagent as depolymerization of the insoluble matrix proceeds. This is consistent with the complete release of glycerol (**6**) esterified in cutin, which is unlikely to be impeded by the nature of the aliphatic monomer. The primary ester linkage between DCA and ω-OHFA will be similar to a primary wax ester linkage, thus slower to react, while the secondary ester linkages between fatty acid monomers and 10,16-dihydroxypalmitate (**5**) will be the least reactive. The methoxide-dependent release of DCA and glycerol > ω-hydroxy fatty acids >10,16-dihydroxypalmitate meets these expectations and points to a significant degree of catenation between 10,16-dihydroxypalmitate (**5**) monomers. A previous study of cork (*Quercus suber*) leaves using hydroxyl group capping has shown that all ω-hydroxyl groups are esterified, whereas 25% the mid-chain hydroxyl groups in 10,16-dihydroxypalmitate (**5**) are esterified (Agullo et al., 1984). Furthermore, in several cutins, 10,16-dihydroxypalmitate (**5**) has been shown to form oligomers with other fatty acid monomers, largely via secondary ester linkages (Graça and Lamosa, 2010; Ray and Stark, 1998; Tian et al., 2008).

In Arabidopsis, ω-OHFA (including 10,16-dihydroxypalmitate (**5**)) could be accommodated as Glycerol-DCA-$(\omega\text{-OHFA})_n$-Glycerol units. A second possibility is that $(\omega\text{-OHFA})_n$ would be esterified directly to other positions on glycerol (**6**). Given the relatively low abundance of ω-OHFA, it is expected that $n = 0 > n = 1 > n =$ larger integers. A third possibility is that DCA-rich cutin is spatially largely separated from the 10,16-dihydroxypalmitate cutin, via either spatial or temporal separation of biosynthesis. Each type of cutin has its own largely distinct set of biosynthetic genes (Li-Beisson et al., 2009; Li et al., 2007a). 10,16-Dihydroxypalmitate (**5**), the major C16 cutin monomer, is preferred by GPAT6 and requires CYP77A6 for synthesis and assembly into cutin (Li-Beisson et al., 2009). The dilution of glycerol-DCA-glycerol motif by additional monomers is expected to be less than indicated by their

stoichiometry. Only one glycerol (**6**) can be attached per ω-OHFA monomer, but it clearly cannot be r**e**tained if catenated (i.e. reacted to produce an estolide). Thus, compared to full monomer release at high NaOMe concentrations to give a glycerol:DCA:ω-OHFA molar ratio of 2:1:0.4, the release of all the glycerol (**6**) and DCA but only half the ω-OHFA at 0.2% NaOMe (i.e. glycerol:DCA:ω-OHFA molar ratio of 2:1:0.2) indicates that at least 80% of the DCA must be in a simple glycerol-DCA-glycerol motif, with no other acyl monomers attached. Because transmethylation may produce a progressively release monomers as the cuticle is depolymerized, this would overestimate the amount of ω-OHFA that actually could be associated with DCA and glycerol (**6**). Despite the uncertainties introduced by 28 mol % ω-OHFA, most of the cutin must retain its glycerol-DCA-glycerol motif.

4. Conclusions

Mild NaOMe-catalyzed transmethylation releases mainly DCA (**1**–**3**) and glycerol (**6**) cutin monomers from the delipidated leaves and stems of Arabidopsis. Using a newly developed isotope dilution method to quantify glycerol (**6**) by GC/MS, the molar stoichiometric ratio of glycerol (**6**) to DCA is 2:1. This ratio is further confirmed by analysis of biosynthetic mutants. It is proposed that this corresponds to a dominant cell wall esterified glycerol-DCA-glycerol structural motif for at least this type of cutin, rather than an extensive aliphatic polyester domain, namely an acylglycerol copolymer, usually presented in models. That is, DCA-rich cutins, which are essential to the formation of stomatal ledges, are true heteropolymers. It will be impossible to develop a full understanding of the genetics-structure-function relationships for this type of cutin, and indeed other classes of cutin, until a more detailed picture of cutin structure is available than at present, and one which explicitly shows how aliphatics connect with the cell wall matrix. The structural motif presented here suggests appropriate biosynthetic and structural experiments. Clearly, this motif does not represent the bulk structures in other types of cutin. Indeed, differential depolymerization rates were observed for different classes of monomers consistent with additional structural motifs for ω-OHFA-rich (**4**, **5**) cutins. The differential reactivity between different ester linkages of model compounds suggests cutin depolymerization can be more precisely refined for future experimentation.

5. Experimental

5.1. Plant materials and growth conditions

All *Arabidopsis thaliana* plants (ecotype Columbia-0) and the mutant lines *gpat4*/*gpat8*, *att1-2* and *lacs2-3*) were grown in pots as described previously (Li et al., 2007a). Seeds of *Brassica napus* cv. Westar were planted in a mixture of soil:vermiculite (2:1 v/v) and grown in a controlled growth chamber (24–25 °C, 60% relative humidity, and a 16/8-hr light/dark cycle and a fluorescent light intensity of 200–250 μmol m^{-2} s^{-1}).

5.2. Delipidation of plant tissues

Seven-week-old Arabidopsis stem and leaf tissues, or 18-day-old/60-day-old *B. napus* leaf tissues, were immersed in boiling *i*-PrOH (25 ml/g fresh wt. tissues) and heated for 10 min at 80 °C. After cooling down to room temperature, the tissues were grounded to fine powders with a Polytron and extracted for 4 h at room temperature in *i*-PrOH. After filtration, the residues were extracted successively with *i*-PrOH (overnight), $CHCl_3$-MeOH (2:1,v/v, 8 h), $CHCl_3$-MeOH (1:2, v/v, overnight), MeOH (8 h), $CHCl_3$-MeOH (1:2, v/v, overnight), $CHCl_3$-MeOH (2:1, v/v, 8 h) and $CHCl_3$ (overnight). All the extraction steps were performed in a shaker at 250 rpm, room temperature and the residues were separated from solvent by filtration through Whatman No. 1 filter paper. The thoroughly delipidated residues were air-dried and placed under vacuum over anhydrous $CaCl_2$ until reaching constant weight before further analysis.

5.3. Isotope dilution gas chromatography mass spectrometry (ID-GC/MS)

Glycerol (≥99.5%) and [$^{13}C_3$]glycerol (99 atom % ^{13}C) were obtained from Sigma-Aldrich and dissolved in MeOH to a final concentration of 1 mg/ml before use. Quantification of glycerol (**6**) by ID-GC/MS was carried out with [$^{13}C_3$]glycerol (99 atom % ^{13}C) as the internal standard. A series of standard mixtures were prepared with a fixed amount of [$^{13}C_3$]glycerol and varying amount of glycerol to (**6**) give a molar ratio range of glycerol to [$^{13}C_3$]glycerol from 0 to 2. The mixtures were acetylated with pyridine (100 μl) and Ac_2O (200 μl) (60 °C, 1hr then room temperature overnight). The reagents were removed under N_2 and dry samples were dissolved in toluene:heptane (0.2 ml, 1:1 v/v) for GC/MS analysis under SIM mode with a dwell time of 0.2 s. Ions at mass 115 and 116, 118 and 119 were selected to monitor triacetin and [$^{13}C_3$*glyceryl*]triacetin, respectively. Calibration curves were generated by plotting relative peak area abundances between labeled and unlabeled ions (i.e. the area ratio of ions 115/118 and ions 116/119) versus the known molar ratio of glycerol:[$^{13}C_3$]glycerol in the range of ratios 0 to 2.

5.4. Simultaneous analysis of glycerol and aliphatics from cutin polyester

Thoroughly dried, delipidated plant tissue residues (ca 0.03 g) were heated at 60 °C for 2 h with occasional vortexing in MeOH (2 ml) containing 15% (v/v) MeOAc and 0.2% (w/v) NaOMe. The MeOAc co-solvent was added to present residual fatty acid saponification if the sample was not thoroughly dried. This may introduce a small amount of hydroxy group acetylation, but it does not matter since there is a downstream per-acetylation reaction (Molina et al., 2006). Methyl heptadecanoate (Sigma, USA), ω-pentadecalactone (Fluka, Switzerland), methyl *cis,cis*-11,14-eicosadienoate (Sigma, USA) and [$^{13}C_3$]glycerol were added as internal standards at 10 μg each. After transmethylation, the reaction mixtures were cooled, neutralized with glacial AcOH and filtered through glass wool. The plant residues were further washed with MeOH (2 ml) and CH_2Cl_2 (2 ml). The filtrates were combined and the solvent removed under N_2. The dried products were acetylated and analyzed by GC/MS on a DB-5 capillary column (J&W Scientific, CA, USA; 30 m × 0.25 mm × 0.25 lm film thickness) with He carrier gas at 1.5 ml/min constant flow and temperature programmed from 100 °C, 5 min, 10 °C/min to 240 °C, then 2 °C/min to 300 °C, and held at 300 °C for an additional 15 min. Splitless injection was used and the mass spectrometer run in scan mode over 40–700 amu (electron impact ionization) as well as SIM mode as mentioned above, with peaks quantified on the basis of their total ion current. A linear total ion current response with respect to mass was obtained over a 0.02–0.4 mg/ml concentration range for methyl heptadecanoate and for C18:1 DCA (**2**) dimethyl diester standards, with <5% difference in mass response between these standards.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.03.017.

References

Agullo, C., Collar, C., Seoane, E., 1984. Free and bound hydroxyl and carboxyl groups in the cutin of *Quercus suber* leaves. Phytochemistry 23, 2059–2060.

Atmodjo, M.A., Hao, Z.Y., Mohnen, D., 2013. Evolving views of pectin biosynthesis. Annu. Rev. Plant Biol. 64, 747–749.

Beisson, F., Li-Beisson, Y., Pollard, M., 2012. Solving the puzzles of cutin and suberin polymer biosynthesis. Curr. Opin. Plant Biol. 15, 329–337.

Bessire, M., Chassot, C., Jacquat, A., Humphry, M., Borel, S., Petetot, J.M.C., Metraux, J.P., Nawrath, C., 2007. A permeable cuticle in Arabidopsis leads to a strong resistance to *Botrytis cinerea*. EMBO J. 26, 2158–2168.

Bonaventure, G., Beisson, F., Ohlrogge, J.B., Pollard, M., 2004. Analysis of the aliphatic monomer composition of polyesters associated with Arabidopsis epidermis: occurrence of octadeca-cis-6, cis-9-diene-1,18-dioate as the major component. Plant J. 40, 920–930.

Chen, X., Truksa, M., Snyder, C.L., El-Mezawy, A., Shah, S., Weselake, R.J., 2011. Three homologous genes encoding *sn*-glycerol-3-phosphate acyltransferase 4 exhibit different expression patterns and functional divergence in *Brassica napus*. Plant Physiol. 155, 851–865.

Christie, W.W., 1993. Preparation of ester derivatives of fatty acids for chromatographic analysis. In: Christie, W.W. (Ed.), Advances in Lipid Methodology-two. Oily Press, Dundee, pp. 69–111.

Deshmukh, A.P., Simpson, A.J., Hatcher, P.G., 2003. Evidence for cross-linking in tomato cutin using HR-MAS NMR spectroscopy. Phytochemistry 64, 1163–1170.

Edwards, S.H., Stribling, S.L., Pyatt, S.D., Kimberly, M.M., 2012. Reference measurement procedure for total glycerides by isotope dilution GC-MS. Clin. Chem. 58, 768–776.

Espelie, K.E., Sadek, N.Z., Kolattukudy, P.E., 1980. Composition of suberin-associated waxes from the subterranean storage organs of seven plants – parsnip, carrot, rutabaga, turnip, red beet, sweet potato and potato. Planta 148, 468–476.

Fang, X.H., Qiu, F., Yan, B., Wang, H., Mort, A.J., Stark, R.E., 2001. NMR studies of molecular structure in fruit cuticle polyesters. Phytochemistry 57, 1035–1042.

Franke, R., Briesen, I., Wojciechowski, T., Faust, A., Yephremov, A., Nawrath, C., Schreiber, L., 2005. Apoplastic polyesters in Arabidopsis surface tissues-A typical suberin and a particular cutin. Phytochemistry 66, 2643–2658.

Gerber, D.W., Byerrum, R.U., Gee, R.W., Tolbert, N.E., 1988. Glycerol concentrations in crop plants. Plant Sci. 56, 31–38.

Graça, J., Lamosa, P., 2010. Linear and branched poly(omega-hydroxyacid) esters in plant cutins. J. Agric. Food Chem. 58, 9666–9674.

Graça, J., Pereira, H., 1997. Cork suberin: a glyceryl based polyester. Holzforschung 51, 225–234.

Graça, J., Pereira, H., 1999. Glyceryl-acyl and aryl-acyl dimers in *Pseudotsuga menziesii* bark suberin. Holzforschung 53, 397–402.

Graça, J., Pereira, H., 2000a. Suberin structure in potato periderm: glycerol, long-chain monomers, and glyceryl and feruloyl dimers. J. Agric. Food Chem. 48, 5476–5483.

Graça, J., Pereira, H., 2000b. Diglycerol alkenedioates in suberin: building units of a poly(acylglycerol) polyester. Biomacromolecules 1, 519–522.

Graça, J., Pereira, H., 2000c. Methanolysis of bark suberins: analysis of glycerol and acid monomers. Phytochem. Anal. 11, 45–51.

Graça, J., Santos, S., 2006. Glycerol-derived ester oligomers from cork suberin. Chem. Phys. Lipids 144, 96–107.

Graça, J., Schreiber, L., Rodrigues, J., Pereira, H., 2002. Glycerol and glyceryl esters of ω-hydroxyacids in cutins. Phytochemistry 61, 205–215.

Jeffree, C.E., 2006. The fine structure of the plant cuticle. In: Riederer, M., Müller, C. (Eds.), Annu. Plant Rev., Biology of the Plant Cuticle, vol. 23 Blackwell Publishing Ltd, Ames, pp. 11–125.

Kolattukudy, P.E., 1981. Structure, biosynthesis, and biodegradation of cutin and suberin. Annu. Rev. Plant Physiol. Plant Mol. Biol. 32, 539–567.

Kügler, X., 1884. Über das Suberin. Inaugurale Dissertation der mathematischen und naturwissenschaftlichen. Fakultät der Kaiser-Wilhelms-Universität, Strassburg, Germany.

Kunst, L., Samuels, A.L., 2003. Biosynthesis and secretion of plant cuticular wax. Prog. Lipid Res. 42, 51–80.

Lequeu, J., Fauconnier, M.L., Chammai, A., Bronner, R., Blee, E., 2003. Formation of plant cuticle: evidence for the occurrence of the peroxygenase pathway. Plant J. 36, 155–164.

Li-Beisson, Y., Pollard, M., Sauveplane, V., Pinot, F., Ohlrogge, J.B., Beisson, F., 2009. Nanoridges that characterize the surface morphology of flowers require the synthesis of cutin polyester. Proc. Natl. Acad. Sci. U. S. A. 106, 22008–22013.

Li, Y., Beisson, F., Koo, A.J., Molina, I., Pollard, M., Ohlrogge, J.B., 2007a. Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. Proc. Natl. Acad. Sci. U. S. A. 104, 18339–18344.

Li, Y., Beisson, F., Ohlrogge, J.B., Pollard, M., 2007b. Monoacylglycerols are components of root waxes and can be produced in the aerial cuticle by ectopic expression of a suberin-associated acyltransferase. Plant Physiol. 144, 1267–1277.

Lü, S.Y., Song, T., Kosma, D.K., Parsons, E.P., Rowland, O., Jenks, M.A., 2009. Arabidopsis *CER8* encodes long-chain acyl-coa synthetase 1 (LACS1) that has overlapping functions with LACS2 in plant wax and cutin synthesis. Plant J. 59, 553–564.

Matzke, K., Riederer, M., 1991. A comparative study into the chemical constitution of cutins and suberins from *Picea abies* (L.) Karst., *Quercus robur* L., and *Fagus sylvatica* L. Planta 185, 233–245.

Moire, L., Schmutz, A., Buchala, A., Stark, R.E., Ryser, U., 1999. Glycerol is a suberin monomer. New experimental evidence for an old hypothesis. Plant Physiol. 119, 1137–1146.

Molina, I., Bonaventure, G., Ohlrogge, J., Pollard, M., 2006. The lipid polyester composition of *Arabidopsis thaliana* and *Brassica napus* seeds. Phytochemistry 67, 2597–2610.

Molina, I., Li-Beisson, Y., Beisson, F., Ohlrogge, J.B., Pollard, M., 2009. Identification of an Arabidopsis feruloyl-coenzyme A transferase required for suberin synthesis. Plant Physiol. 151, 1317–1328.

Molina, I., Ohlrogge, J.B., Pollard, M., 2008. Deposition and localization of lipid polyester in developing seeds of *Brassica napus* and *Arabidopsis thaliana*. Plant J. 53, 437–449.

Pollard, M., Beissson, F., Li, Y., Ohlrogge, J.B., 2008. Building lipid barriers: biosynthesis of cutin and suberin. Trends Plant Sci. 13, 236–246.

Ray, A.K., Stark, R.E., 1998. Isolation and molecular structure of an oligomer produced enzymatically from the cuticle of lime fruit. Phytochemistry 48, 1313–1320.

Riederer, M., Schonherr, J., 1988. Development of plant cuticles: fine structure and cutin composition of *Clivia miniata* Reg. leaves. Planta 174, 127–138.

Schmidt, H.W., Schonherr, J., 1982. Development of plant cuticles: occurrence and role of non-ester bonds in cutin of *Clivia miniata* Reg. leaves. Planta 156, 380–384.

Schmutz, A., Buchala, A.J., Ryser, U., 1996. Changing the dimensions of suberin lamellae of green cotton fibers with a specific inhibitor of the endoplasmic reticulum-associated fatty acid elongases. Plant Physiol. 110, 403–411.

Schmutz, A., Jenny, T., Amrhein, N., Ryser, U., 1993. Caffeic acid and glycerol are constituents of the suberin layers in green cotton fibers. Planta 189, 453–460.

Schnurr, J., Shockey, J., Browse, J., 2004. The acyl-CoA synthetase encoded by *LACS2* is essential for normal cuticle development in Arabidopsis. Plant Cell 16, 629–642.

Stark, R.E., Yan, B., Ray, A.K., Chen, Z., Fang, X., Garbow, J.R., 2000. NMR studies of structure and dynamics in fruit cuticle polyesters. Solid State Nucl. Magn. Reson. 16, 37–45.

Suh, M.C., Samuels, A.L., Jetter, R., Kunst, L., Pollard, M., Ohlrogge, J., Beisson, F., 2005. Cuticular lipid composition, surface structure, and gene expression in Arabidopsis stem epidermis. Plant Physiol. 139, 1649–1665.

Tian, S.Y., Fang, X.H., Wang, W.M., Yu, B.W., Cheng, X.F., Qiu, F., Mort, A.J., Stark, R.E., 2008. Isolation and identification of oligomers from partial degradation of lime fruit cutin. J. Agric. Food Chem. 56, 10318–10325.

Villena, J.F., Dominguez, E., Stewart, D., Heredia, A., 1999. Characterization and biosynthesis of non-degradable polymers in plant cuticles. Planta 208, 181–187.

Watson, J.T., 1990. Selected-ion measurements. Methods Enzymol. 193, 86–106.

Welti, R., Li, W.Q., Li, M.Y., Sang, Y.M., Biesiada, H., Zhou, H.E., Rajashekar, C.B., Williams, T.D., Wang, X.M., 2002. Profiling membrane lipids in plant stress responses – role of phospholipase D alpha in freezing-induced lipid changes in Arabidopsis. J. Biol. Chem. 277, 31994–32002.

Xiao, F., Goodwin, S.M., Xiao, Y.M., Sun, Z.Y., Baker, D., Tang, X.Y., Jenks, M.A., Zhou, J.M., 2004. *Arabidopsis CYP86A2* represses *Pseudomonas syringae* type III genes and is required for cuticle development. EMBO J. 23, 2903–2913.

Yan, B., Stark, R.E., 2000. Biosynthesis, molecular structure, and domain architecture of potato suberin: a ^{13}C NMR study using isotopically labeled precursors. J. Agric. Food Chem. 48, 3298–3304.

Yang, W., Pollard, M., Li-Beisson, Y., Beisson, F., Feig, M., Ohlrogge, J., 2010. A distinct type of glycerol-3-phosphate acyltransferase with *sn*-2 preference and phosphatase activity producing 2-monoacylglycerol. Proc. Natl. Acad. Sci. U. S. A. 107, 12040–12045.

Yang, W., Simpson, J.P., Li-Beisson, Y., Beisson, F., Pollard, M., Ohlrogge, J.B., 2012. A land-plant-specific glycerol-3-phosphate acyltransferase family in Arabidopsis: substrate specificity, *sn*-2 preference, and evolution. Plant Physiol. 160, 638–652.